DETECTION OF *CANDIDA*

FIELD OF THE INVENTION

The present invention relates to a method and a
5 means of diagnosing *Candida* infection. In particular the
present invention relates to a method of diagnosing
Candida infection which is both sensitive and rapid.

BACKGROUND OF THE INVENTION

10 *Candida* is the most commonly identified causative
agent of oral or vaginal thrush. However, over the last
few decades *Candida* has emerged as a significant cause of
life-threatening infections in hospital patients.
Ironically the increasing incidence of these "invasive" or
15 "systemic" *Candida* infections has been advances in modern
medicine. Patients that are now surviving major injuries,
surgery, cancers and organ transplants are vulnerable to
life-threatening *Candida* infections. In the United
States, *Candida* is now the forth most common cause of
20 blood infections in hospitals.

The major problem with systemic *Candida*
infections is that there are few definitive clinical signs
or symptoms. Treatment is largely based on suspicion
rather than a definitive diagnosis. Even with the
25 availability of anti-fungal drugs such as fluconazole a
high mortality rate (30 to 70%) is associated with
systemic *Candida* infections. The high rate of mortality
is largely due to the rapid onset of infection and a
rapidly fatal outcome. Without an accurate diagnosis the
30 infection often goes unnoticed until it is too late to
effectively treat. This has led to a comment by
clinicians that *Candida* infections are usually diagnosed
at autopsy. Accordingly, there is a need for a rapid
diagnostic assay that is capable of early diagnosis of
35 *Candida* infection so that appropriate treatment may be
instituted thereby reducing the mortality rate.

The main difficulty in the diagnosis of *Candida*

infections is that being a commensal, mere isolation of *Candida* from body surfaces, or orifices, is not diagnostic of an infection. Culture of *Candida* from blood or deep tissue is still the main method of diagnosis of systemic

5   *Candida* infections. However, it can take several days for a culture to become positive, by then it may be too late to effectively treat the infection. Also, false positives may occur due to contamination from superficial body sites. Of more importance, is the observation that in up

10   to fifty percent of autopsy proven cases of systemic candidiasis, blood cultures were negative and therefore of no diagnostic value.

Nuclear magnetic resonance (NMR) and radioisotope scanning have been used to detect *Candida* infections in

15   tissues and organs. However, those methods are not useful for early diagnosis.

Recently analysis of the *Candida* metabolite arabinitol was proposed as a diagnostic tool. However, as arabinitol is produced by the human body, further clinical

20   studies have cast doubt on its value.

The polymerase chain reaction (PCR) has also been used in the diagnosis of invasive *Candida* infections. However, PCR has not established itself as a useful diagnostic method for *Candida* for the same reasons as

25   outlined above ie *Candida* is a ubiquitously present microorganism and false positives, due to superficial contamination, are prevalent.

Immunoassays are the established procedures for the diagnosis of many types of infectious diseases.

30   Immunoassays have the advantage that they are rapid and have a standardised assay format. Immunoassays can be designed to either detect *Candida* antigens, or host antibodies reactive against *Candida* antigens. Several immunoassays are commercially available for the detection

35   of *Candida* antigens in sera or other body fluids. However, these assays lack either sensitivity or specificity or both.

Immunoassays have been developed based on the detection of immunodominant *Candida* antigens. *Candida* mannan is a highly immunogenic cell wall antigen. However, as *Candida* is a commensal, most individuals have antibody to *Candida* mannan, so its usefulness in the diagnosis of systemic infection is limited. The applicant has now surprisingly found that a more discriminatory assay for *Candida* than previously used is the detection of cytoplasmic antigen. The advantage of this diagnostic assay is that antibody to this cytoplasmic antigen is only produced in response to an actual infection. The applicant has further demonstrated that the use of a combination of cytoplasmic antigens with other antigens is very predicative of *Candida* infection.

Accordingly, the present invention overcomes or at least alleviates the problems normally associated with diagnosing *Candida* infection.

SUMMARY OF THE INVENTION

In its most general aspect, the invention disclosed herein provides a simple and rapid method for diagnosis of *Candida* infection. The method of diagnosis of *Candida* infection may be used to screen large numbers of samples for possible infection.

Accordingly, in one aspect, the invention provides a method of diagnosing *Candida* infection, comprising the steps of:

a). obtaining a biological sample from a subject at risk of, or suspected to be suffering from, *Candida* infection, and

b). measuring the levels of antibody to *Candida* cytoplasmic antigen present in the biological sample.

Antibody levels may be measured using known techniques of immunology including enzyme-linked immunoassay (ELISA or EIA), biligand binding (sandwich technique), fluorometric assay, chemiluminescent assay, radialimmunodiffusion or radioimmunoassay (RIA). ELISA or

chemiluminescent assay methods are particularly preferred, since these are quick, sensitive, and specific, and are readily automated for large-scale use. These methods also provide quantitative determinations.

5   The diagnostic method utilises antigens expressed by *Candida*, especially cytoplasmic antigen. The antigens isolated from *Candida* as disclosed herein may, in certain embodiments of the diagnostic method of the present invention, be immobilised on an inert surface, embedded in

10   a gel, or may be conjugated to a molecule which imparts colour, fluorescence or radioactivity to the antigen.

In a second aspect, the invention provides a method for assessing the prognosis of *Candida* infection, comprising the steps of measuring the levels of antibody

15   to *Candida* cytoplasmic antigen in a biological sample.

Persons skilled in the art will appreciate that the techniques disclosed herein may be used on any type of biological sample. Preferable the biological sample is selected from the group consisting of bone marrow, plasma,

20   spinal fluid, lymph fluid, the external sections of the skin from respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood; both whole blood and sera, blood cells, tumours and organs. Most preferably the biological sample is sera.

25   Biological samples that may be analysed by the method of the present invention can also be obtained via swabs, shunts or the like. The biological samples may be analysed directly, or may be treated prior to testing by, for example, concentration or pH adjustment.

30   In a third aspect, the present invention further provides a method of detecting the presence or absence of a *Candida* antibody comprising the steps of:

a). exposing a biological sample, which may include a *Candida* antibody, to an isolated cytoplasmic

35   *Candida* antigen; and

b). detecting the reaction between antibody and antigen.

In an especially preferred embodiment of the present invention the diagnostic assay further utilises other *Candida* antigens in combination with the cytoplasmic antigen. In particular the cell wall antigen (including mannose) and/or purified immunodominant antigen (enolase) are utilised.

Accordingly, in a forth aspect of the present invention there is provided a method of diagnosing *Candida* infection, comprising the steps of:

a). obtaining a biological sample from a subject at risk of, or suspected to be suffering from, *Candida* infection, and

b). measuring the levels of antibody present in the biological sample to *Candida* cytoplasmic antigen in combination with measuring the levels of antibody to either cell wall antigen or immunodominant antigen (enolase) or both.

The reagents and means of diagnosis of the present invention may also be embodied in a kit for use in a diagnostics laboratory or may be adapted and automated for analysing large numbers of samples at a central receiving centre.

Accordingly, in a fifth aspect the invention provides a kit when used for detecting the presence or absence of a *Candida* antibody in a biological sample, comprising:

a). a biological sample collection device;

b). a cytoplasmic *Candida* antigen; and

c). means for detecting reaction between the antibody and antigen in the sample.

Suitable buffering agents and ionic salts may also be included in the kit.

In a sixth aspect the invention provides a method of preparing a cytoplasmic antigen comprising the step of removing lipoproteins by chloroform extraction.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a coomassie blue stained SDS-PAGE with major protein bands of the *Candida* cytoplasmic antigen fraction observed at 55kDa, 35 to 45kDa region, 30kDa and 20kDa.

Figure 2 shows a single coomassie blue band of 48kDa corresponding to the expected size of the enolase antigen.

Figure 3 shows a coomassie blue stained gel of the clarified cell wall antigen preparation. A broad smear of stain can be seen ranging in size from 90kDa to 200kDa

Figure 4 shows a number of sera screened against the *Candida* cytoplasmic antigen preparation.

Figure 5 shows antibody reactivity to the three Candida antigens - cytoplasmic, cell wall and immunodominant antigens, using negative control sera.

Figure 6 shows antibody reactivity to the three Candida antigens - cytoplasmic, cell wall and immunodominant antigens, using sera from patients with superficial candidiasis.

Figure 7 shows antibody reactivity to the three Candida antigens - cytoplasmic, cell wall and immunodominant antigens, using sera from patients with systemic candidiasis.

ABBREVIATIONS USED

EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
RIA	Radioimmunoassay
BSA	Bovine serum albumin
DMSO	Dimethyl sulfoxide
β-Me	β-mercaptoethanol
TMB	3,3',5,5'-tetramethyl-benzidine

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The practice of the present invention employs, unless otherwise indicated, conventional molecular biology, cellular biology, and immunoassay techniques

5   within the skill of the art. Such techniques are well known to the skilled worker, and are explained fully in the literature. *See*, e.g., Harlow and Lane, "Antibodies: A Laboratory Manual" (1988); Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1982); "Animal

10 Cell Culture" (R.I. Freshney, ed., 1986); "Immobilised Cells and Enzymes" (IRL Press, 1986); B. Perbal, "A Practical Guide to Molecular Cloning" (1984); Sambrook, et al., "Molecular Cloning: a Laboratory Manual" (1989) and Ausubel, F. et al., 1989-1999, "Current Protocols in

15 Molecular Biology" (Green Publishing, New York).

In describing the present invention, the following terminology is used in accordance with the definitions set out below.

As used herein, a "biological sample" refers to a

20 sample of tissue or fluid isolated from a individual, including but not limited to bone marrow, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood; both whole blood and

25 anti-coagulated whole blood, blood cells, tumours, organs, and also includes samples of *in vivo* cell culture constituents, including but not limited to conditioned medium resulting from the growth of cells in cell culture medium, putatively *Candida* infected cells, recombinant

30 cells, and cell components.

"Human tissue" is an aggregate of human cells which may constitute a solid mass. This term also encompasses a suspension of human cells, such as blood cells, or a human cell line.

35 For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word

"comprises" has a corresponding meaning.

It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any
5   of these documents forms part of the common general knowledge in the art, in Australia or in any other country.

Persons skilled in the art will appreciate that any number of different immunoassays may be used in the
10   present invention. For example, the *Candida* antigens disclosed herein may be used in antibody capture assays, antigen capture assays, wherein the antigen/antibody complex forms a "special" class of antigen or two-antibody sandwich assays.
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TECHNIQUES USED FOR ANTIGEN PREPARATION

The term "*Candida* antigen" as used here means any one of the three separate types of *Candida* antigen utilised in the present invention, namely, cell wall
20   antigen (including mannose), total cytoplasmic antigen (mannose depleted) or purified immunodominant antigen (enolase). Use of the term "*Candida* antigens" means that all three antigens were involved or could be utilised. A number of techniques may be used to prepare the *Candida*
25   antigens including biochemical extraction, column chromatography, Gel fractionation, gene cloning, differential precipitation, filtration, dialysis or centrifugation; however, the preferred techniques are those disclosed herein. Briefly, these techniques involve
30   either mechanical, chemical or enzymatic lysis of *Candida* cells, followed by separation of insoluble cell walls from soluble cytoplasmic fraction by centrifugation, filtration and dialysis. Chemical treatment of cell wall fraction to release cell wall antigens followed by centrifugation and
35   dialysis. Filtration and organic extraction of soluble cytoplasmic cell extract. Separation of mannoproteins by ConA affinity chromatography. Purification of the

immunodominant enolase antigen from the soluble
cytoplasmic extract by anion and cation affinity
chromatography. It will be appreciated by those skilled
in the art that other techniques, or modifications or
5   variations of the above techniques, may be adopted without
adversely affecting the spirit of the present invention.

TECHNIQUES USED FOR ANTIBODY PREPARATION AND LABELLING

Antiserum to the *Candida* antigens disclosed
10 herein may be produced in a host animal such as rabbit or
sheep. The serum fraction containing the antibody may be
isolated by standard techniques. This antiserum may be
employed in several of the embodiments of the invention
hereinafter set forth, or a more sensitive and specific
15 antibody might be obtained by further purification of the
serum by electrophoresis, high-speed centrifugation or the
like. Ultimately, large quantities of highly specific
monoclonal antibody may be produced by means of the
hybrid-myeloma techniques by methods known to those
20 skilled in the art.

Certain embodiments of the present invention
employ antibody to the *Candida* antigens immobilised on
cellulose, agarose, sephadex or glass beads or other
similar inert surfaces such as metal, plastic or ceramic
25 which do not interfere with subsequent reaction.
Adsorption, Br-CN activation or other techniques known in
the art may be employed to immobilise the antibody.

Other embodiments of the present invention employ
the antibody to the *Candida* antigens conjugated to a
30 chromophoric (highly coloured) molecule, an enzochromic
(an enzyme which produces colour upon addition of
reagents) molecule, fluorochromic (fluorescent) molecule
or a luminogenic (luminescent) molecule.

The conjugate of antibody with enzyme is made
35 using techniques known in the prior art. (For references,
see Avrameas, S. and Uriel, J., in Comptes Rendus
Hebdomadaires des Seances de l'Academie des Sciences, vol.

262, p. 2543, (1966); Nakane, P.K. and Pierce, G.B., in Journal of Histochemistry and Cytochemistry, vol. 14, p. 929, (1966); Nakane, P.K., in Methods in Enzymology, vol. 37, p. 133, (1975)).

5   Chromophoric molecules that may be used are 2,3-dinitrobenzene (DNB) salts, dinitrophenol (DNP) and methyl and butyl orange. Other suitable chromophoric agents are well known in the art. Enzochromic molecules that may be conjugated with the antibody are enzymes that give colour

10   with appropriate reagents. Examples are alkaline phosphatase (ALP) which develops colour with nitrophenyl phosphate (NPP), glucose oxidase with glucose, and D-galactopyranoside. These and other examples are well known in the art. Examples of fluorogenic agents are 2,4-

15   dinitrofluorobenzene and pipsyl derivatives. Luminogenic molecules may be conjugated to antibodies by the method of Branchini, et al. (Biochem. Biophys. Res. Commun. 97, 334 [1980]). The term chromophoric hereinafter is intended to include enzochromic ,

20   fluorochromic and luminogenic molecules as well.

      Certain embodiments of the invention also utilise *Candida* antibody tagged with a radioactive element. I^{125} conjugated by means of the chloramine-T procedure is a common example, but other methods known in the art may

25   also be employed.

TECHNIQUES USED FOR ANTIGEN IMMOBILISATION AND LABELLING

      Antigen molecules may be immobilised on a solid carrier by a variety of methods known in the art,

30   including covalent coupling, direct adsorption, physical entrapment and attachment to a protein-coated surface. For references describing the methodology, see Silman, I. H. and Katchalski, E. in Annual Review of Biochemistry, Vol. 35, p. 873 (1966); Melrose, G. J. H., in Review of

35   Pure and Applied Chemistry, Vol. 21, p. 83, (1971); and Cuatrecasas, P. and Anfinsen, C. B., in Methods in Enzymology, Vol. 22, (1971).

Lai et al. (German OS No. 2,539,657, U.S. Pat. No. 4,066,512) discloses a method of attachment to a protein-coated surface. In this method, the internal and external surfaces of a microporous membrane are first

5 coated with a water-insoluble protein such as zein, collagen, fibrinogen, keratin, glutelin, polyisoleucine, polytryptophan, polyphenylalanine, polytyrosine, or copolymers of leucine with p-amino phenylalanine. Such a coating renders the membrane capable of immobilising a

10 wide variety of biologically active proteins including enzymes, antigens, and antibodies. A microporous structure is defined as one having more than 50% of its total volume in the form of pores ranging in size from 25 nanometres to 25 micrometers, preferably from 25

15 nanometres to 14 micrometers. A pore size range from 25 nanometres to 5 micrometers is employed in most applications herein. Uncoated microporous membranes have as much as 70 to 75% of their volume as pore space. The pores permit liquid flow through the membrane. After

20 being coated by zein, for example, the pore space is reduced 5 to 10% with the result that the structure retains its essential properties of having a high proportion of its volume as pore space and permitting liquid flow through the pores. The structure has a large

25 surface area in contact with any solution contained within the pores.

Such a coated membrane, having immobilised antigen or antibody, provides a compact, easy to manipulate carrier for the immobilised antigen or

30 antibody. Its integral structure permits removal of bound from unbound components by simple mechanical means.

Non-specific binding may be minimised by interposing a second stage immobilisation step, in which an immunochemically neutral protein is immobilised to the

35 filter. Immobilisation therefore occurs in two stages according to a preferred embodiment of the invention: a first stage in which the desired immunochemical component

is immobilised, and a second stage, following the completion of the first, in which an immunochemically neutral protein such as fetal calf serum or bovine gamma globulin is next immobilised. The term immunochemically neutral is defined in terms of the specific components of the assay. Any protein, which does not combine immunochemically with a component of the assay or with one of the reagents, is considered immunochemically neutral, even though such protein might be immunochemically reactive in another system.

Where the substance to be detected is an antibody, the immunochemically reactive moiety of the conjugate must be an antibody capable of binding immunochemically with the antibody to be tested. Such antibodies may be obtained by immunising an animal with the antibody or immunoglobulin fraction of serum from the animal in which the antibody to be tested originated. For example, where the antibody to be tested is a human antibody, a goat antibody against human antibody is obtained from the serum of a goat immunised against human immunoglobulin (antibody). The enzyme moiety may be any enzyme capable of catalysing a reaction which can be detected by any method known to those skilled in the art, and which retains its activity after conjugation with antibody. Horseradish peroxidase is preferred because of its convenience and suitability to a wide range of applications. It is well known that the enzyme catalyses the oxidations of a variety of organic compounds in the presence of hydrogen peroxide. Many such organic substrates are chromogenic, ie. undergo a colour change upon oxidation.

It has been found in the present invention that the purity of the enzyme preparation used in the formation of conjugate has an effect on the degree of non-specific binding. The greater the purity of the enzyme preparation, the less the non-specific binding. In part, the reduction is made possible because, the total amount

of conjugate protein required is reduced as the specific
activity of the enzyme is increased. The opportunity for
non-specific binding is therefore reduced as well. In the
preferred embodiment, the use of a highly purified

5   peroxidase preparation has been found to significantly
reduce the amount of colour reaction observed in control
samples as compared with known positives.

TECHNIQUES USED FOR *CANDIDA* ANTIBODY DETECTION

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Antibody Capture Technique

         A *Candida* antigen prepared by the techniques
disclosed herein is immobilised, preferably on an inert
surface such as PVC, paper or a similar bibulous mat. The

15   immobilised *Candida* antigen is then put into contact with
a sample suspected of containing *Candida* antibody. In the
case of aqueous samples such as blood or urine, the
solution is buffered and ionic salts may be present at
optimum concentration for *Candida* antibody-*Candida* antigen

20   interaction. TRIS or borate buffered phosphate at pH 7.5
to 9.0 and ionic strength about 0.010 to 0.5, for example,
are suitable buffering agents and ionic salts. The inert
surface with *Candida* antigen or *Candida* antigen-*Candida*
antibody complex thereon is next put into contact with

25   antibody to *Candida* antigen conjugated to a chromophormic
molecule. Preferably the *Candida* antigen is in solution
buffered at pH from about 7.5 to 9.0 and ionic
concentration equivalent to about 0.01M to about 0.1M
NaCl. After careful rinsing under water or with suitable

30   surfactants such as Tween 20 to remove excess coloured
antibody, the inert surface is inspected for colour,
fluorescence or luminescence directly or after addition of
colour-developing agents. Colour on the inert surface
indicates interaction between immobilised *Candida* antigen-

35   *Candida* antibody complex in solution. A control may be
run for colour comparison.
         This technique may be adapted to clinical use by

employing *Candida* antigens tagged with radioactive
elements and observing either depletion of activity in
solution or uptake on solid support of radioactivity.
This embodiment is highly sensitive and rapid and suitable

5   for large numbers of samples.

Enzyme-Linked Immunoassay-ELISA

              A solution comprising *Candida* antibody conjugated
to enzyme which forms colour with developing reagents and
10   buffer and ionic salts suitable for reaction between
Candida antigen and the *Candida* antibody is put into
contact and allowed to react with *Candida* antigen
immobilised, preferably, on an inert surface such as PVC,
paper strip or glass bead. The amount of enzochromic
15   conjugated *Candida* antibody is sufficient to saturate
about 50% of the reactive sites on the immobilized
antibody. The inert surface with antibody-*Candida* antigen
enzyme complex is put into contact with buffered sample
suspected of containing *Candida*, said sample having an
20   unknown amount of *Candida* antibody. The colour of the
resultant immobilised antibody-*Candida* antigen-enzyme
complex on the strip after colour developing reagents are
added is observed in comparison to a control strip which
has not been treated with sample containing *Candida*
25   antibody. Dilution in colour on inert surface treated
with sample means presence of *Candida* antibody in the
unknown sample.
              This method may he adapted for clinical use by
contacting samples and immobilised enzyme, preferably in
30   tubes which may be centrifuged and watching developing
colour spectrophotometrically. This embodiment is very
sensitive and rapid.

Radialimmunodiffusion-Precipitin Reaction

35   One of the *Candida* antigens is suspended in a
softened gelatinous medium such as agar or agarose along
with buffers and salts to maintain pH between about 6.0 to

9.0 and ionic strength between about 0.01M to 0.5M for optimal antigen-antibody interaction. The suspending medium of U.S. Pat. No. 4,259,207 is a suitable example. The mixture is spread out to harden on a test plate or,

5 preferably, poured into a disc-shaped container such as an Octolony plate. A small amount of sample is placed on the solidified gel, preferably in a centre well and the plate or disc is allowed to stand preferably covered for a period of hours. Diffusion of sample into the surrounding

10 area occurs during this period. If the *Candida* antibody is present, it reacts with the embedded *Candida* antigen and causes an opaque area in a radial pattern about the point of application of sample. A control can be run for comparison. Calibration of an amount of *Candida* antibody

15 in the sample, if desired, can be obtained by controlling temperature, time and size of sample and comparing the resultant size of radial area with one of known concentration.

20 Radioimmunoassay

A *Candida* antigen of the present invention is immobilised on an inert surface such as glass beads in a separation column. A portion of *Candida* antigen is conjugated to a radioactive element, preferably I^{125} and

25 allowed to react with the immobilised *Candida* antigen in an amount sufficient to, saturate 50% of the binding sites. The immobilised *Candida* antigen-enzyme complex is put into contact with a sample suspected of containing *Candida* antibody, the sample being buffered between pH 6-9

30 and containing total ionic salts about 0.05 to 0.5M for optimal reaction conditions for formation of *Candida* antigen-antibody complex. The *Candida* antibody is eluted from the antigen and the eluant is measured for radioactivity. Loss of activity compared to a control

35 indicates *Candida* antibody in the sample.

Haemagglutination

     Candida antibody may be assayed through standard haemagglutination techniques with *Candida* antigen to antibody used as sensitising agent.

5   It is to be understood that methods described hereinabove for assay of *Candida* antibody employing coloured reagents have been presented most specifically for application where neither trained personnel nor sophisticated instruments are available. These methods, 10 however, may be adapted for use in a clinical setting where large numbers of samples are to be assayed by substituting radioactive elements for chromogenic conjugated molecules.

     It is also to be understood that the term 15 "colour" is not to be interpreted as being limited to the narrow visible range of the electromagnetic spectrum, but is meant to include wavelengths which may be measured by standard spectrophotographic instruments such as spectrophotometers and absorption and emission 20 colourimeters in both the uv and the ir range.

     Although it is contemplated that the methods of the present invention are to be applied to biological fluids themselves, the sensitivity and specificity of the method can be improved by culture of the fluids preferably 25 on medium selective for *Candida* prior to testing.

     Sensitivity may also be improved by preliminary treatment of biological samples with lysing agents such as isotonic solution, sound, or lysozyme to release *Candida* antibody into the extracellular environment. U.S. Pat. No. 30 4,166,765, for example, discloses suitable lysing procedures for biological samples containing bacteria. Any lysing agent may be employed which does not interfere with subsequent enzyme activity.

35 ASSAYS EMBODIED IN KIT FORM

     The diagnostic method and means of the present invention may be embodied in the form of a kit for use by

individuals for self-diagnosis of *Candida* in the privacy of their homes.

The kit comprises a means for sample collection, the *Candida* antigen to *Candida* antibody and a means for
5   detecting reaction between sample and *Candida* antigen.

In embodiments adapted for clinical use, electrophoretic separation techniques such as isoelectric focusing or zone electrophoresis which are based on differences of both size and charge distribution between
10   products and reactants may likewise be used to separate products from reactants. Products separated electrophoretically may be detected by characteristic locations compared to standards or may be identified by colour or immunochemically. Resinous beads of charged
15   surfaces may also be used to separate products and reactants.

The means for detecting reaction in the case of immunoassay in a preferred embodiment of the invention is a gelatinous medium in which the *Candida* antigen to
20   antibody is suspended. The gelatinous medium is in a transparent glass or plastic container and comprises buffer and ionic salts for optimal conditions for formation of the *Candida* antigen-antibody complex. Reaction is noted as a transparent area radiating from the
25   central point at which the sample is applied.

The means for detecting reaction in another preferred embodiment comprising immunoassay is the *Candida* antigen to *Candida* antibody conjugated to a chromophore in a sealed, sterile packet along with buffer and ionic
30   salts. For assay, the contents of the packet are diluted with water in a marked tube supplied in the kit. Included also in this embodiment is the antigen to *Candida* antibody immobilised on an inert surface. For assay, the inert surface with immobilised *Candida* antigen is put into
35   contact with sample and then with the solution of chromophore-conjugated *Candida* anti-IgA antibody, protein A or protein G. The inert surface is inspected for

colour, which indicates *Candida.*

Although the invention has been described with reference to presently preferred embodiments, it should he understood that various modifications can be made without

5   departing from the spirit of the invention. Moreover, the following examples are offered by way of illustration only and are not intended to limit the invention in any manner. All patent and literature references cited herein are expressly incorporated.

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EXAMPLE 1   PREPARATION OF *CANDIDA* ANTIGEN

The following three types of *Candida* antigen were prepared:

1). Cell wall antigen (including mannose);

15   2). Total cytoplasmic antigen (mannose depleted); and

3). Purified immunodominant antigen (enolase).

A clinical isolate of the *Candida albicans,* was obtained from a patient with vaginal thrush. The identity

20   of the *Candida* species was confirmed with the use of an API 20C Auxonagram strip (API System S.A., France). The *C. albicans* isolate was designated KEMH5.

200ml YEPD culture medium (1% yeast extract, 2% peptone, 2% D-glucose) was inoculated with the isolate as

25   a starter culture and incubated for 24h at 30°C with aeration. The starter culture was then used to inoculate a 10L YEPD culture incubated under similar conditions in a 23L Bio-Flo Fermenter IV System (New Brunswick Scientific, Edison, NJ).

30   The *Candida* culture was harvested from the Bio-Flo fermenter system and separated from culture medium with the use of a Pellicon filtration cassette (Millipore, USA). Concentrated cells were separated from residual medium by centrifugation in 500ml centrifuge flasks for

35   15min at 1,660 x g and 4°C. The supernatant was discarded and the pelleted cells were resuspended in protein extraction buffer (20mM bis-Tris, pH 6.5). The yeast

cells were then centrifuged as described previously, resuspended and pooled for further processing.

    Candida cells were ruptured mechanically with the use of a Dynomill® (WAB, Switzerland). Milling was
5 continued until 99% cell disruption was obtained. The soluble *Candida* cell extracts were collected and dispensed into 50ml centrifuge tubes. The extracts were centrifuged for 12h at 8,517 x g and 4°C to precipitate insoluble cell walls. The supernatants containing the soluble
10 cytoplasmic antigen fraction were recovered and passed through a 0.45μm filter membrane.

    The filtrates were then extracted with an equal volume of chilled chloroform. Following centrifugation at 4°C for 15min at 1,036 x g the upper aqueous phase was
15 aspirated and transferred to a dialysis tube. The soluble cytoplasmic protein fractions were dialysed in column binding buffer (20mM Tris/HCl, pH 7.4, 0.5M NaCl, 1mM $MnCl_2.4H_2O$, 1mM $CaCl_2$) for 12h in preparation for chromatography.

20 The soluble cytoplasmic antigen fraction was depleted of contaminating soluble cell wall mannoprotein by Con A-Sepharose chromatography. The dialysed cytoplasmic antigen fraction was filtered through a 0.45μm filter. 50ml of the dialysed extract was applied onto a
25 Con A-Sepharose column (2.6 x 12.5 cm) equilibrated in binding buffer at a flow rate of 4ml/min. The unbound flow-through fraction (non-glycosylated proteins) was collected. Bound mannoproteins were eluted with 0.5M α-methyl mannoside in binding buffer. This step was
30 performed before the next run and to clean the column before storage.

    The soluble cytoplasmic antigen fraction was dialysed overnight against 20mM Tris.Cl, pH7.4. An estimate of the quantity of protein in solution was
35 performed using the Bio-Rad® (Bradford) microassay procedure in accordance with the manufacturers instructions. A portion of the cytoplasmic antigen

- 20 -

extract was analysed by SDS-PAGE.

As shown in Figure 1 there was a number of major
protein bands observed which varied in size from approx
20kDa up to approx 60kDa in size. The major staining
5   bands being at 55kDa, four bands in the 35 to 45kDa
region, 30kDa and 20kDa. This was in stark contrast to
the large number of Coomassie blue staining bands in the
original crude lysate prior to organic extraction and Con
A-Sepharose chromatography.

10   Purification of the enolase antigen was conducted
in the same fashion as the soluble *Candida* cytoplasmic
antigen except that it was not subjected to Con
A-Sepharose chromatography. Instead, following dialysis
and filtering through a 0.20µm syringe filter (cellulose
15   acetate), the filtered extracts were applied to a
Pharmacia Biotech XK 50/20 chromatography column packed
with Pharmacia Biotech Source 15Q quaternary ammonium
anion exchanger (Pharmacia LKB, Uppsala, Sweden). The
column was equilibrated prior to chromatography with
20   column binding buffer 'A' (20mM bis-Tris, pH 6.5). Anion
exchange chromatography of the crude extracts was
controlled and recorded using the Bio-Rad® Econo® system
(Bio-Rad Laboratories, USA). Bound protein was eluted
from the column with a salt gradient of buffer 'B' (1M
25   NaCl in buffer 'A', pH 6.5). The recovered fractionated
proteins were analysed by an enzyme activity assay.

The active enzyme enolase hydrolyses D(+)2-
phosphoglyceric acid (PGA) to phosphoenolpyruvate (PEP).
The production of PEP can be monitored by
30   spectrophotometry at 240nm. 20µl of protein solution was
combined with 1ml of enolase substrate solution (50mM
Tris-HCl pH 7.4, 2.7mM magnesium acetate, 1.0mM EDTA,
1.2mM D(+)2-phosphoglyceric acid) in a quartz cuvette and
the change of absorbance recorded at 1min intervals. The
35   specific activity was defined as the conversion of 1µmol of
PGA to PEP per min per mg protein. An estimate of the
quantity of protein in solution was performed using the

Bio-Rad® (Bradford) microassay procedure.

Eluate fractions containing enolase activity were selected and dialysed for 12h at 25°C in hpH$_2$O. The dialysed fractions were recovered and filtered through a
5   0.20μm syringe filter. The filtrate was concentrated ten-fold by evaporation under vacuum for 5h. The concentrated samples were dialysed with binding buffer 'A' (10mM sodium acetate, pH 4.7) immediately prior to application to a Pharmacia Biotech Mono S HR10/10 chromatography column
10   packed with methyl sulphonate cation exchanger (Pharmacia LKB, Uppsala, Sweden). Cation exchange chromatography was performed using the Bio-Rad® Biologic system. Bound protein fractions were eluted from the column with a salt gradient of buffer 'B' (1M NaCl in buffer 'A', pH 4.7).
15   Fractions containing enolase activity were identified by the enzyme activity assay described above.

Figure 2 shows a single Coomassie blue band of 48kDa corresponding to the expected size of the enolase antigen. The identification of the 48kDa antigen as the
20   glycolytic enzyme enolase was confirmed by an enolase activity assay.

Purification of the cell wall antigen was conducted as follows: the precipitated insoluble cell walls were collected following centrifugation as described
25   above. The cell walls were washed with hpH$_2$O then collected by centrifugation at 6,000 rpm. This step was repeated three times or until the supernatant was no longer cloudy. This ensured any residual soluble cytoplasmic antigen was removed from the cell wall
30   preparation. The washed cell wall pellet was then resuspend in 10mM Phosphate buffer pH7.4 containing 1% v/v β-Me and incubated for 30min at 37°C in a shaker to solubilise the cell wall antigens. The sample was then centrifuged for 5min at 8,000 rpm and the pellet was then
35   discarded. The supernatant was transferred into a fresh tube and recentrifuged (5min at 8,000 rpm). The supernatant containing the solubilised cell wall antigen

was then dialysed in hpH$_2$O for 48h at 4°C (four changes of water), or until no odour was detected. Following dialysis the sample was centrifuged three times 5min at 8,000 rpm to remove any residual particular matter.

5   Following clarification the cell wall antigen preparation was analysed by SDS-PAGE. The resulting Coomassie blue stained gel is presented in Figure 3. A broad smear of stain is seen ranging in size from 90 kDa to 200 kDa. The lack of discrete protein bands is typical

10 of mannoproteins, where differences in the number of mannose groups added to the protein base results in a variety of molecular weights.

EXAMPLE 2   ENZYME LINKED IMMUNOSORBENT ASSAYS (ELISAS)

15   A serum panel was collected from 1998 to 2000 from various patients with *Candida* infections. Negative control (Control) sera (n=20) were obtained from the Red Cross Blood Bank, Perth, Australia and was obtained from healthy males in the 19 to 25 year age group. Sera (n=13)

20 from patients with recurrent vulvo vaginal candidiasis (VVC) were obtained from King Edward Memorial Hospital, Perth, Australia. Sera (n=108) from patients with oral candidiasis were obtained from Clinipath Ltd and the UWA Dental School, Perth, Australia. Sera (n=39) from

25 patients (n=28) with systemic candidiasis were obtained from Princess Margaret Hospital, Perth, Australia and Prince of Wales Hospital, Sydney, Australia.

          In the case of patients with oral and vaginal *Candida* infection, confirmation of infection was made by

30 physical examination and by culture of *Candida* organisms from the relevant body site. In the case of patients with systemic infection, confirmation of infection was through positive blood culture or biopsy. In all cases the immune status of the patient was unknown.

35   Sera from patients with either superficial or systemic candidiasis were screened by ELISA using trays coated with the *Candida* cytoplasmic antigen. The protein

content of each antigen preparation was determined using a commercial assay (BioRad) with BSA as a standard. A series of ELISAs were performed to determine the optimal coating concentration for each antigen (data not shown).

5   The optimal coating concentration being that which gave the greatest discrimination between a positive and a negative control serum. For each antigen the optimum coating concentration was determined to be 2μg/ml.

A 96 well C8 strip microtitre plate (Greiner

10 GmbH, Germany), was coated with either *Candida* cell wall antigen, cytoplasmic antigen, or purified enolase antigen as prepared in Example 1. 50μl of a 2.0μg/ml solution of the antigen was diluted in coating buffer (0.1M NaHCO$_3$, pH 9.3) and added to individual wells. The plates were

15 incubated for 12h at 4°C then equilibrated to ambient temperature. After equilibrating the plates to ambient temperature, coating solution was decanted and the plate tapped dried. Plates were inverted on paper towel to drain. Alternatively excess coating solution was

20 aspirated by the automated plate washer (Dynatech Laboratories, Chantilly VA, USA). It was important not to wash the plate at this stage.

A volume of 300μl of blocking solution (PBS pH 7.3, 2% (w/v) BSA (ICN, Australia), 0.01% (w/v) Tween 20),

25 was applied to each well and incubated at 25°C for 90min. Blocking solution was decanted and the plate tapped dried. Plates were inverted on paper towel to drain and tapped dried for a second time. At this stage plates were either used immediately, or dried for storage. Plates to be

30 dried were placed inverted in a sealable container such as a plastic food container with a number of silica gel desiccant sachets for 48h. The inclusion of approximately 20 small desiccant sachets was adequate for the drying of 6 coated ELISA micro-well trays. Dried plates were sealed

35 into heat-sealed packets with a single desiccant sachet and labelled. Plates were stored at 4°C until required. Packets containing plates were equilibrated to ambient

temperature before opening.

Human test sera diluted 1/100 in blocking solution was dispensed into wells in 50µl aliquot's and incubated at 37°C for 30min. The primary antibody solution was aspirated and wells were washed six times in PBS-Tween 20. The plates were inverted on paper towels and allowed to drain for 10min. The plates were then tapped dried.

A volume of 100µl of a horseradish peroxidase anti-human IgG conjugate diluted 1/10,000 in blocking solution was dispensed to each well. Secondary antibody solution was incubated at 37°C for 30min. The secondary antibody solution was aspirated and wells were washed six times in PBS-Tween 20. Plates were inverted on paper towel to drain for 10min and then tapped dried. Plates were inverted on paper towel for a second time and allowed to drain for 5min. Plates were then tapped dried. Particular care was employed to ensure that all traces of secondary conjugate solution was removed as residual conjugate was established as the major factor responsible for disparity of results (Dynatech Laboratories Inc, USA).

A volume of 100µl of TMB liquid substrate solution was dispensed into each well and developed at 25°C for 10min. The reaction was terminated with the addition of 100µl of 1M phosphoric acid or 1M H_2SO_4. The absorbance values for each well were measured at 450nm, reference 620nm with a MRX automated plate reader.

Each immunoassay was performed in triplicate and the mean value of absorbance was used. The absorbances are shown as a Scatter diagram in Figure 4. Three groups of patients with *Candida* infections were analysed. The first group were patients with systemic candidiasis (Systemics), the second group had oral candidiasis (Oral) and the third group had vulvovaginal candidiasis (VVC). Blood bank sera (Control) from males in the 19 to 25 year age group, who were at low risk of having an undetected or subclinical *Candida* infection were used as a control. The cut-off absorbance (OD_{450} = 0.22) was the mean value of the

negative control sera. From these data the cytoplasmic antigen ELISA had a sensitivity of 89% and a specificity of 95%. This is higher than that reported for other *Candida* serological tests (Zoller et al., 1991. J. Clin.

5   Micro. 29:1860-1867).

To further increase the sensitivity of the *Candida* ELISA multiple antigens were used. These were the cell wall, cytoplasmic and native enolase (described above).

10   The use of multiple antigens increased the sensitivity of the *Candida* ELISA. It also provided greater discrimination between superficial and systemic infection. Six negative control sera (serum obtained from healthy males in the 19 to 25 year age group) were used in

15   ELISAs with microtitre tray wells individually coated with the three *Candida* antigens. For each serum the antibody titre to each of the three antigens was below that of the cut-off line (Figure 5). This line is the cut off value assigned based on a comparison of the average antibody

20   titres of sera from control patients versus those of candidiasis patients. The value plotted on the y-axis of the graph is the ratio of the cut-off absorbance divided into the absorbance of the test serum.

Serum obtained from 6 patients with superficial

25   candidiasis was then reacted in the ELISA. Again the absorbance value of each serum was divided by the absorbance of the cut-off (Figure 6). The characteristic antibody response of the sera from patients with superficial candidiasis was a high titre against the cell

30   wall antigen preparation (1.5 to 2 times the cut-off value). The antibody reactivity to the complete cytoplasmic antigen preparation was positive in most cases (1 to 1.5 times the cut-off). In contrast the antibody titre to the enolase antigen was below or equal to that of

35   the cut-off. There is a correlation between the antibody titre to the internal *Candida* antigens (cytoplasmic and enolase) and the severity of the superficial infection

(data not shown). However, the severity of the infection in the six patients analysed was not known.

Six sera taken from patients with systemic candidiasis (confirmed by positive blood culture) were
5   analysed by ELISA. The results are presented in Figure 7. In the case of the patients with systemic candidiasis the antibody response to the cell wall antigen preparation was positive (1.5 to 2 times the cut-off value). Also, the antibody titres to the internal *Candida* antigens
10   (cytoplasmic and enolase) were also positive (1.5 to 2.5 times cut-off value).

CONCLUSIONS

The *Candida* mannan depleted cytoplasmic antigen
15   preparation disclosed herein can be used to identify patients with *Candida* infections. The sensitivity and specificity using an ELISA with microtitre trays coated with this antigen is greater than that obtained by other *Candida* diagnostic tests. Further, the ELISA assay format
20   disclosed herein is easier to perform, more robust and more rapid than formats used in other available *Candida* diagnostic assays. The ELISA format also has the advantage that it is quantifiable. This enables the patient to be monitored over a period of time and changes
25   in the titre of the antibody response to the *Candida* antigens recorded. The ability of the test to monitor overtime the antibody titre to *Candida* antigens has a prognostic value in terns of measuring the patient's response to antifungal drugs and in the overall survival
30   prospects of the patient. Another advantage of the cytoplasmic antigen preparation is that the method developed to produce the antigen is simpler and more rapid than other available procedures (eg. compare with that of Zoller et al., 1991, *supra*).